

Molecular Analysis of Insertion/Deletion Mutations in Protein 4.1 in Elliptocytosis

I. Biochemical Identification of Rearrangements

in the Spectrin/Actin Binding Domain and Functional Characterizations

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Abstract

Protein 4.1 (80 kD) interacts with spectrin and short actin filaments to form the erythrocyte membrane skeleton. Mutations of spectrin and protein 4.1 are associated with elliptocytosis or spherocytosis and anemia of varying severity. We analyzed two mutant protein 4.1 molecules associated with elliptocytosis: a high molecular weight 4.1 (95 kD) associated with mild elliptocytosis without anemia, and a low molecular weight 4.1 (two species at 68 and 65 kD) associated with moderate elliptocytosis and anemia. 4.1⁹⁵ was found to contain a ~15-kD insertion adjacent to the spectrin/actin binding domain comprised, at least in part, of repeated sequence. 4.1^{68/65} was found to lack the entire spectrin-actin binding domain. The mechanical stability of erythrocyte membranes containing 4.1⁹⁵ was identical to that of normal membranes, consistent with the presence of an intact spectrin-actin binding domain in protein 4.1. In contrast, membranes containing 4.1^{68/65} have markedly reduced mechanical stability as a result of deleting the spectrin-actin binding domain. The mechanical stability of these membranes was improved following reconstitution with normal 4.1. These studies have thus enabled us to establish the importance of the spectrin-actin binding domain in regulating the mechanical stability of the erythrocyte membrane. (*J. Clin. Invest.* 1990. 86:516–523.) Key words: erythrocytes • protein 4.1 • elliptocytosis • mutations

Introduction

The erythrocyte membrane is composed of a lipid bilayer, transmembrane proteins which include the glycophorins and band 3, the anion channel, and a membrane skeleton. The major components of the membrane skeleton are spectrin, protein 4.1 (hereafter referred to as 4.1), ankyrin, and short actin filaments, all of which interact to form an extended network underlying the lipid bilayer. Specific associations between ankyrin and band 3 and between 4.1 and glycophorin attach the skeletal network to the lipid bilayer. The membrane skeleton confers upon the red cell its shape and the properties

of deformability and mechanical stability necessary for its 120-d lifespan in the microcirculation. The concept that the membrane skeleton plays an important role in determining shape and deformability of the red cell has been supported by discoveries in recent years of deficiencies and mutant forms of spectrin, 4.1, and ankyrin in erythrocytes of patients with hereditary spherocytosis and elliptocytosis.

Protein 4.1 deficiency was first reported in three children of a consanguineous marriage who had transfusion-dependent hemolytic elliptocytosis (HE)¹ (1, 2). Erythrocytes of the affected children were entirely lacking in 4.1, while the parents had partial deficiency of 4.1 and mild elliptocytosis. Erythrocyte membranes lacking 4.1 were found to exhibit markedly decreased mechanical stability (2). Reconstitution of 4.1-deficient red cells with purified 4.1 by exchange hemolysis restored normal stability to the 4.1-deficient membranes (3). A subsequent study by Alloisio et al. (4) of 10 families from southeastern France and North Africa with typical mild elliptocytosis revealed partial 4.1 deficiency in seven affected members of four families.

It has long been recognized that HE is inherited in linkage with Rh blood group phenotype in a subset of families (5). This association has now been explained by assignment of the 4.1 gene to chromosome 1 in close proximity to the genes for Rh blood group types (6). Agre and colleagues (7) have described three distinct variants of erythrocyte 4.1 associated with HE that are inherited in linkage with the Rh blood group type. Affected members of family C of Italian ancestry have erythrocytes with reduced content of 4.1 of normal molecular weight (~80 kD). Affected members of family N, of Scottish-Irish descent, are heterozygous for a high molecular weight form of 4.1 at ~95 kD (4.1⁹⁵). Affected members of family G, of Italian descent, are heterozygous for normal 4.1⁸⁰ and two low molecular weight forms of 4.1 at ~68 and 65 kD (4.1^{68/65}). In this family, the presence of 4.1^{68/65} is associated with moderately severe hemolytic elliptocytosis. We describe here the site and nature of the insertion resulting in 4.1⁹⁵, the deletion(s) resulting in 4.1^{68/65}, and the functional consequences of these mutations. The mutant 4.1 proteins were examined by chemical and enzymatic cleavages originally used by Leto and Marchesi (8) to characterize normal 4.1. In a companion paper, the mutations are further characterized by sequencing of PCR-amplified mRNA around regions of cDNA determined from the protein data to be the sites of insertion or deletion.

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1. *Abbreviations used in this paper:* DFP, diisopropylfluorophosphate; HE, hemolytic elliptocytosis; HMW, high molecular weight; IEP, isoelectric point; LMW, low molecular weight; NTCB, nitrothiocyanobenzoic acid.

Methods

Extraction and purification of protein 4.1.

Red cells were washed, lysed, and white ghosts were dialyzed at low ionic strength (0.1 mM EDTA, 0.1 mM diisopropylfluorophosphate, DFP, pH 9.0, at 4°C) to remove spectrin, as previously described (8). The spectrin-depleted vesicles were homogenized and suspended in 5 mM NaH₂PO₄, 1 mM EDTA, 1 M KCl, 1 mM DFP, and 0.05 mM phenylmethylsulfonylfluoride (PMSF), pH 7.5, incubated overnight at 4°C and then at 37°C for 30 min to extract 4.1, ankyrin (band 2.1) and band 6. The extract was centrifuged at 100,000 g, and the supernatant was dialyzed against the same buffer but with KCl concentration reduced to 20 mM. Protein 4.1 was separated from ankyrin and band 6 by chromatography on Whatman DE 52 (Whatman Inc., Clifton, NJ) using a linear KCl gradient of 20 to 300 mM KCl in 5 mM NaH₂PO₄, 1 mM EDTA, 0.5 mM β -mercaptoethanol, pH 7.6. The purified 4.1 was dialyzed into 5 mM NaH₂PO₄, 1 mM EDTA, and 200 mM KCl.

Proteolytic cleavages of protein 4.1

Cysteine-specific cleavage of protein 4.1 was performed as previously described (8). Briefly, intact IOVs or 4.1 electroeluted from acrylamide gels were incubated in 7.5 M guanidine HCl, 0.2 M Tris, 1 mM EDTA, at pH 8.0 in the presence of nitrothiocyanobenzoic acid (NTCB) at 0.225 μ g/ μ l (for 4.1 extracts) or 0.6 μ g/ μ l (for IOVs) at room temperature for 1 hr. The pH was then adjusted to 9.0 with Tris base and the samples were incubated overnight at 37°C. The reaction was stopped with 10 mM Tris, 1.0 mM β -mercaptoethanol, pH 8.0, and lyophilized.

Chymotryptic digestion of purified 4.1 was performed in 10 mM Tris HCl, 1.0 mM β -mercaptoethanol, pH 8.0 for 30 min at 2°C at ratios of chymotrypsin:4.1 ranging from 1:100 to 1:500 (8). The digestion was stopped by the addition of 1 mM DFP and lyophilization.

Cyanogen bromide (CNBr) cleavage of purified, lyophilized 4.1 (150–200 μ g) was performed in 70% formic acid containing 200 mol CNBr per mol methionine in 4.1 and incubated for 24 h at 37°C in the presence of argon. The digests were then diluted 1:20 with distilled water and lyophilized.

Membrane protein analysis

Electrophoresis in one dimension of red cell ghosts and NCTB peptides was performed on SDS-PAGE according to Laemmli (9) followed by staining with Coomassie blue or transfer to nitrocellulose membranes for immunoblotting. Two-dimensional electrophoresis (isoelectric focusing-SDS-PAGE, IEF/SDS) was performed as described for spectrin tryptic peptides (10), except that the focusing gel was run in the reverse direction (acid to base).

Anti-4.1 antibodies. Antibodies to five synthetic peptides of 4.1, ranging in size from 15 to 26 residues and encompassing the four chymotryptic domains of 4.1 were raised in New Zealand white rabbits (see Fig. 1). All antibodies generated were affinity-purified on Sepharose-conjugated to the appropriate peptide antigen.

Immunoblots. Peptides of 4.1 resulting from enzymatic or chemical cleavages were electrophoresed in one or two dimensions, transferred to nitrocellulose (Schleicher and Schuell, Inc., Keene, NH) and blotted with polyclonal (rabbit) antibody generated either to intact 4.1 or to synthetic peptides derived from one of the four chymotryptic domains of 4.1, incubated with ¹²⁵I-Staph A protein (Amersham Corp., Arlington Heights, IL, and Pharmacia Fine Chemicals, Piscataway, NJ), and exposed to Kodak XAR film for times varying from 3 h to 1 wk.

NH₂-terminal sequence determinations. One- or two-dimensional SDS gels of chymotryptic peptides of 4.1 were prepared with recrystallized SDS and transferred to Immobilon® membranes (Polyvinylidene difluoride; Millipore Corp., Bedford, MA) in 25 mM Tris, 190 mM glycine, 20% methanol at 50 V for 16 to 24 h. The membranes were stained with Coomassie blue for 5 min, destained, dried, and stored at -20°C. NH₂-terminal sequence was performed on selected peptides cut from these membranes.

Membrane mechanical stability measurements

Resealed membranes for mechanical stability measurements were prepared by a procedure adapted from Johnson (11). The erythrocytes were washed three times in 5 mM Tris, 140 mM NaCl (pH 7.4), and then lysed in 40 vol of 7 mM NaCl and 5 mM Tris (pH 7.4). The membranes were then pelleted by centrifugation, resuspended in 10 vol of 5 mM Tris and 140 mM NaCl (pH 7.4) and incubated 30 min at 37°C for resealing.

For mechanical stability measurements, 100 μ l of a 40% membrane suspension was mixed with 3 ml dextran (40,000 mol wt, 35 g/100 ml in 10 mM phosphate buffer, pH 7.4, viscosity 95 centipoise) and subjected continuously to 750 dyn/cm² in the ektacytometer (12). Under this stress, the membranes progressively fragment, generating undeformable spheres. This process is detected as a time-dependent decrease in the DI. The time required for the DI to fall to 60% of its maximum value is termed T₆₀ and is taken as a measure of membrane stability.

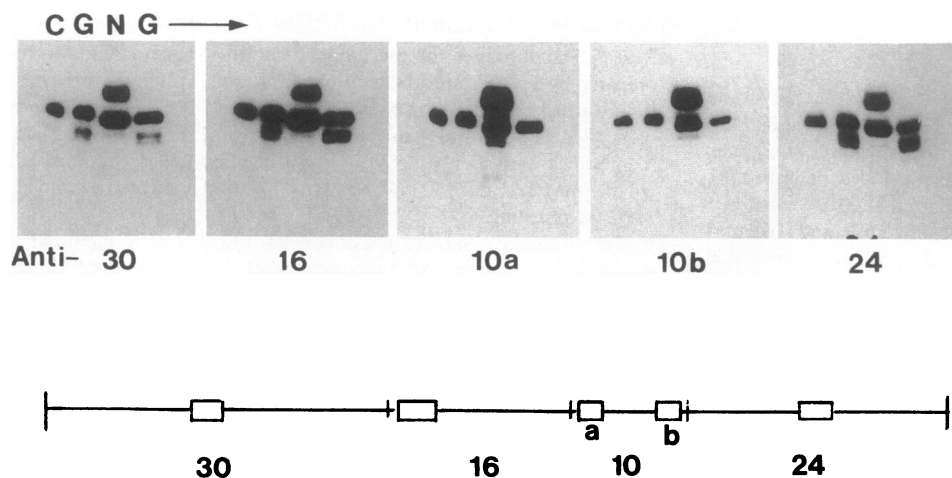
Protein 4.1 reconstitution experiment

To incorporate purified normal protein 4.1 into the membranes with mutant protein 4.1, we modified the technique of exchange hemolysis described by Clark and Shohet (13). In brief, elliptocytic erythrocytes were first washed with the isotonic sodium phosphate buffer (pH 7.4, 290 mosmol/kg) and then lysed in 40 vol of ice-cold hypotonic sodium phosphate buffer (pH 7.4, 40 mosmol/kg). The membranes were collected by centrifugation at 39,000 g for 2 min at 0°C. Four-tenths milliliter of purified protein 4.1 solution of a defined concentration was added to 0.4 ml of packed membranes. The mixture was gently stirred at 0°C for 20 min. Following this incubation in the cold, 0.1 ml of a mixture of KCl, MgCl₂ and dithiothreitol (DTT) was added to the membrane suspension to give a final concentration of 100 mM KCl, 1 mM MgCl₂ and 1 mM DTT. The membranes were then incubated at 37°C for 60 min to allow them to reseal. Incorporation of protein 4.1 into the protein 4.1-deficient membranes was confirmed by SDS-PAGE analysis of the reconstituted membranes.

Results

Purification of 4.1 from erythrocytes of affected members of family N with elliptocytosis yielded a 4.1-species of ~ 95 kD and normal 4.1⁸⁰ in nearly equal proportions, as previously described by McGuire et al. (7). The high molecular weight (HMW) 4.1 reacts with all five antibodies generated to synthetic peptides of the four chymotryptic domains (Fig. 1), suggesting that the high molecular weight (HMW) species contains all normal domains of 4.1 plus an insertion of ~ 15 kD. We will henceforth refer to the 4.1 of affected members of family N as 4.1⁸⁰⁺⁹⁵ and to the isolated HMW 4.1 as 4.1⁹⁵.

NTCB Peptides of 4.1⁸⁰⁺⁹⁵. Nitrothiocyanobenzoic acid (NTCB) cleaves proteins at cysteine residues. Since the seven cysteine residues in protein 4.1 are clustered with the NH₂-terminal chymotryptic 30 kD domain (8), comparison of 4.1⁸⁰ and 4.1⁹⁵ NTCB peptides should indicate whether or not the insertion resulting in 4.1⁹⁵ is NH₂-terminal. Proteins 4.1⁸⁰ and 4.1⁹⁵ were electroeluted from acrylamide gel slices and cleaved with NTCB as described in Methods. NTCB peptides of 4.1⁸⁰ and 4.1⁹⁵ were immunoblotted with antibody to intact 4.1 and to a synthetic peptide of the NH₂-terminal 30 kD domain. The short NH₂-terminal NTCB peptides of 4.1⁸⁰ and 4.1⁹⁵ were identical in size, while the larger (COOH-terminal) NTCB peptides of 4.1⁹⁵ from a ladder ~ 15 kD larger than those of 4.1⁸⁰ (data not shown). The conclusion from this approach was that the insertion resulting in 4.1⁹⁵ is not NH₂-terminal, nor is it within the NH₂-terminal 30 kD chymotryptic domain.



(KKKRERLDGENIYIRC); 10b peptide (MESVPEPRDSEWDKC); and 24 kD peptide (GVLLTAQTITSETPSSTTTTQITKC).

Figure 1. Red cell ghosts from a control (C:4.1⁸⁰), patient N (heterozygous for high molecular weight form of protein 4.1:4.1⁸⁰⁺⁹⁵), and patient G (heterozygous for low molecular weight forms of protein 4.1:4.1^{80+68/65}), immunoblotted with antibody to synthetic peptides from each of the chymotryptic domains, shown in the model below. The amino acid sequence of the synthetic peptides of chymotryptic domains of 4.1 to which polyclonal antibodies were raised is as follows: 30 kD peptide (GSYTIQSELG-DYDPELHGVDYVSDC); 16 kD peptide (TQAQTRQASALIDR-PAPHFERC); 10a peptide

Chymotryptic peptides of 4.1⁸⁰ and 4.1⁹⁵. Chymotryptic cleavage of 4.1 at enzyme:substrate ratios of ~ 1/500 to 1/200 occurs primarily at three sites, producing four major peptides as described by Leto et al. (8) and shown in Fig. 2, *a* and *b*. Proceeding from the NH₂ terminus, these peptides are as follows: (*a*) a basic 30-kD fragment containing all cysteines in 4.1; (*b*) a 16-kD peptide of intermediate isoelectric point; (*c*) an 8- to 10-kD peptide containing the spectrin-actin binding site and a site of phosphorylation by cAMP-dependent protein kinase (4, 14–16); and (*d*) an acidic ~ 24 kD COOH-terminal peptide. Under the mild conditions of digestion used, chymotryptic cleavage at the three sites described is incomplete, re-

sulting in the presence of overlapping peptides at ~ 34 kD (24 + 10), ~ 46 kD (30 + 16), ~ 50 kD (24 + 10 + 16), and ~ 56 kD (30 + 16 + 10) (8).

Fig. 2 *a* shows two-dimensional maps of chymotryptic peptides of 4.1⁸⁰ and 4.1⁸⁰⁺⁹⁵ immunoblotted with anti-4.1 antibody, and a diagrammatic model of the chymotryptic domains of 4.1. Both peptide maps show 24- and 34-kD peptides with similar acidic isoelectric points. In addition, the chymotryptic map of 4.1⁸⁰⁺⁹⁵ has a new 44-kD peptide with the same isoelectric points (IEP) as the normal 34-kD peptide, and a new 60-kD peptide with the same IEP as the normal 50-kD peptide.

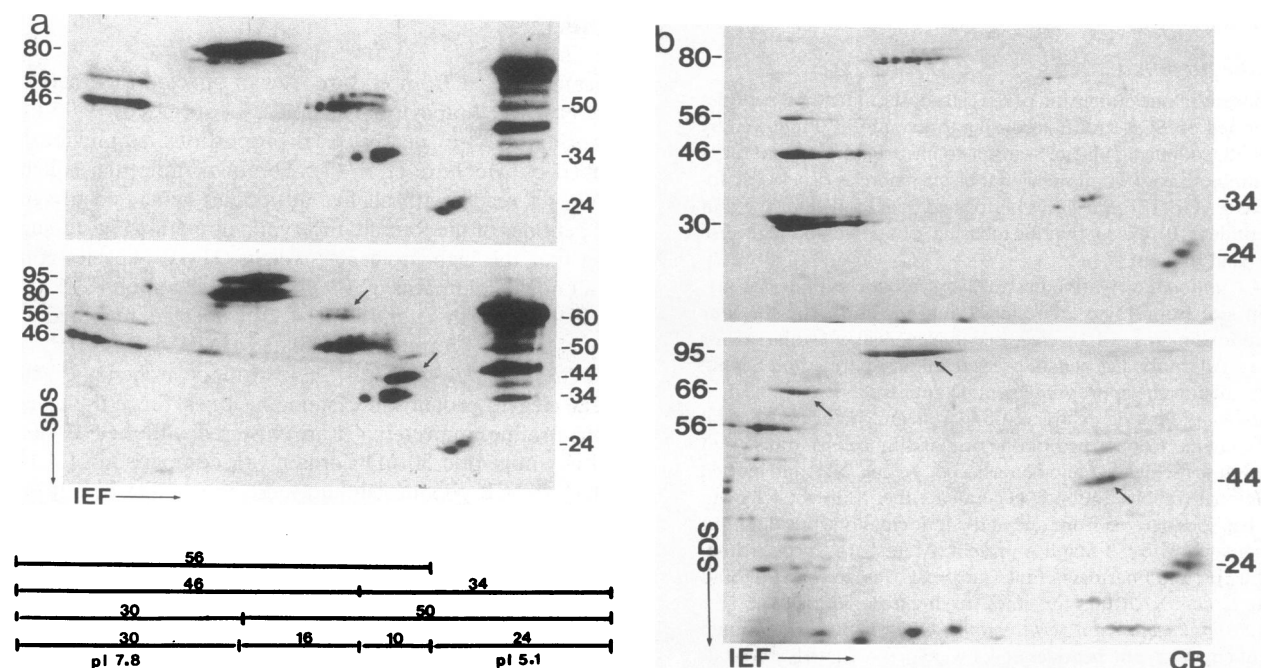


Figure 2. (*a*) Two-dimensional maps of the chymotryptic peptides of 4.1⁸⁰ (*top*) and 4.1⁸⁰⁺⁹⁵ (*bottom*): immunoblot of chymotryptic maps with antibody to 4.1. Arrows mark peptides unique to 4.1⁹⁵. Chymotryptic peptides of normal 4.1 are shown in the models below. Numbers indicate apparent kD of 4.1 peptides. (*b*) Two-dimensional (IEF/SDS) maps of chymotryptic peptides of 4.1⁸⁰ (*top*) and 4.1⁹⁵ (*bottom*) stained with Coomassie blue. Numbers indicate apparent kilodaltons of peptides. Arrows mark peptides unique to 4.1⁹⁵.

Fig. 2 *b* compares Coomassie blue-stained chymotryptic maps of normal 4.1⁸⁰ and 4.1⁹⁵ isolated by electroelution from acrylamide gel slices. The digest of 4.1⁹⁵ has the normal acidic 24-kD peptide, lacks the normal 34-kD peptide, and demonstrates the new 44-kD peptide seen in Fig. 2 *a*. 4.1⁹⁵ also has a new ~66-kD basic peptide which is thought to represent an addition of ~10 kD to the normal 56-kD peptide. Taken together, the results of NTCB and chymotryptic cleavage of 4.1⁹⁵ indicated that the inserted sequence resulting in 4.1⁹⁵ is neither NH₂-terminal nor COOH-terminal, but that it is adjacent to the 10-kD actin-spectrin binding domain and that the new 44, 60, and 66 chymotryptic peptides in 4.1⁹⁵ contain part or all of the insert. (See linear model of 4.1 in Fig. 2 *a*.)

Cyanogen bromide (CNBr) peptides of 4.1⁸⁰ and 4.1⁸⁰⁺⁹⁵. In an attempt to localize more precisely the insert resulting in 4.1⁹⁵, normal and mutant 4.1 were digested with cyanogen bromide (CNBr), which cleaves at methionine residues. The 10-kD chymotryptic domain has two methionines, and it was anticipated that the size of CNBr peptides generated from normal and mutant 4.1 and their reactivity with antibodies to the 10 kD domain would help to determine the site of insertion in 4.1⁹⁵. Fig. 3 shows two-dimensional maps of CNBr peptides of 4.1⁸⁰ and 4.1⁸⁰⁺⁹⁵ immunoblotted with antibodies to synthetic peptides from the NH₂- and COOH-terminal regions of the 10-kD domain, termed 10a and 10b, respectively. The diagram below the figure compares chymotryptic and CNBr peptide domains, and locates 10a and 10b peptides. The CNBr peptides of 4.1⁸⁰⁺⁹⁵ include a cluster of basic peptides at ~12–17 kD not present in normal 4.1 digests that are reactive with antibody to 10a but not to 10b, suggesting that the insertion producing 4.1⁹⁵ is NH₂-terminal to the normal 10-kD domain, or that CNBr cleavage at the methionine residue within the 10b synthetic peptide interferes with its recognition by the anti-10b antibody.

An attempt to determine NH₂-terminal sequence of unique 4.1⁹⁵ CNBr peptides was unsuccessful, presumably due to chemical damage of their NH₂-termini. However, extensive chymotryptic digestion of 4.1⁸⁰⁺⁹⁵ generated a peptide with size and IEP comparable to the 12 kD CNBr peptides, one which is not found in similar digests of 4.1⁸⁰ (data not shown). The NH₂-terminal four residues of this unique 12-kD peptide (Fig. 4) are EAAQ, identical to the last four residues of the first exon within the 24 kD domain of normal 4.1⁸⁰ (17, 18). These four residues are followed by 20 residues of the 10-kD spectrin-actin binding domain, beginning at a known splice site for the first exon in the 10 kD region: *KKRER ([17–19] see Fig. 4). The sequence EAAQ in the intact 24 kD domain is normally preceded by a tryptophan, consistent with the observed chymotryptic cleavage at that site.

The NH₂-terminal sequence of the 44-kD peptide unique to 4.1⁹⁵ was found to be identical to the NH₂-terminus of the 24-kD domain (Fig. 4). These data suggested that the insertion producing 4.1⁹⁵ is the result of partial repeat of the 24-kD domain, and possibly of the 10-kD domain (see Discussion).

Characterization of mutant low molecular weight 4.1^{68/65}. Fig. 5 shows partially purified 4.1 from patient G, originally described by McGuire and Agre (7). In addition to 4.1⁸⁰, there are two low molecular weight (LMW) species of ~68 and ~65 kD which together comprise less than half of the total 4.1 present. Normal protein 4.1⁸⁰ from patient G reacts with all five antibodies generated to synthetic peptides of the four chymotryptic domains (Fig. 1). The LMW species of 4.1 from patient G (4.1^{68/65}) reacts with antibodies to the 30-, 16-, and 24-kD domains (Fig. 1), but shows no reaction with two antibodies to the 10-kD domain (10a and 10b), suggesting that the entire spectrin-actin binding domain is missing.

Chymotryptic peptides of 4.1^{68/65}. The boundaries of the deletion in 4.1^{68/65} were investigated in a manner similar to

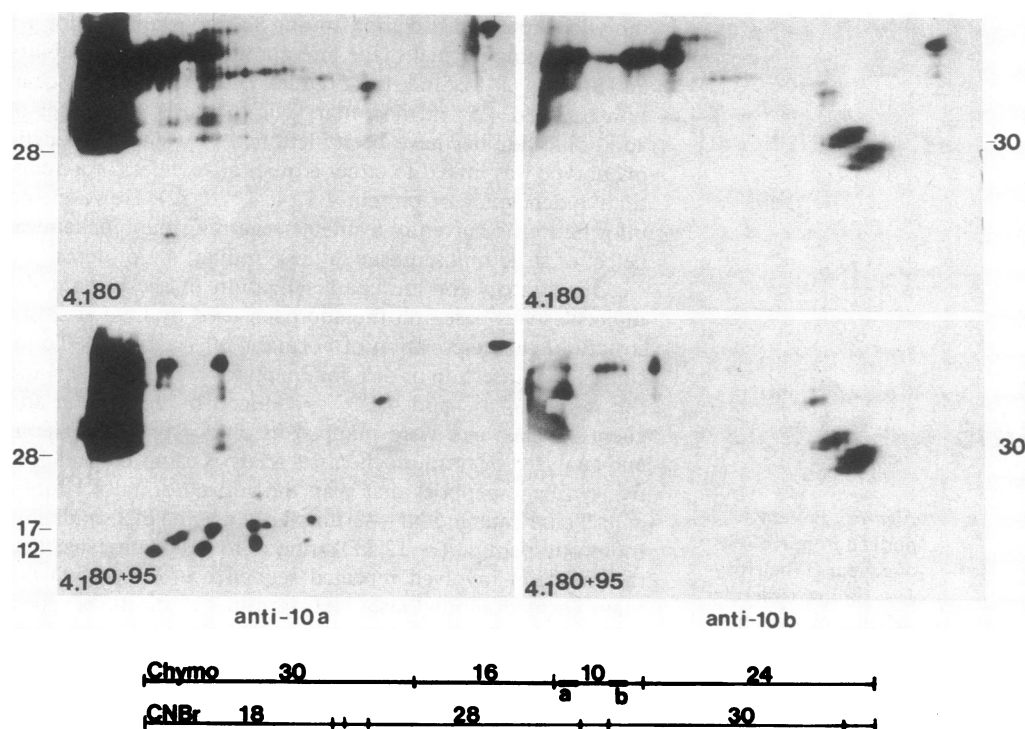


Figure 3. Two-dimensional maps of cyanogen bromide (CNBr) peptides of 4.1⁸⁰ and 4.1⁸⁰⁺⁹⁵ immunoblotted with antibody to synthetic peptides of the 10-kD chymotryptic domain termed 10a and 10b. The short vertical lines in the models below indicate sites of cleavage of 4.1 by chymotrypsin and CNBr, and identifies antigens 10a and 10b.

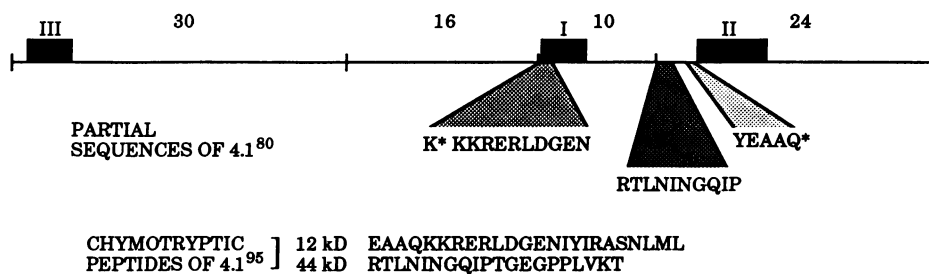


Figure 4. NH₂-terminal sequence of 12 and 44 kD chymotryptic peptides unique to 4.1⁹⁵. The position of related sequence of 4.1⁸⁰ are shown with respect to the normal chymotryptic map and to the known exon structure. exon ■, *splice sites, 30, 16, 10, 24, chymotryptic domains.

those used to study 4.1⁹⁵. Chymotryptic peptides of 4.1^{80+68/65} electrophoresed in two dimensions and immunoblotted with antibodies to the 16- and 24-kD chymotryptic domains (Fig. 6) show the normal 24-, 34-, and 50-kD peptides as well as new peptides of ~ 40 kD (circled) not present in normal 4.1 maps. The new 40-kD peptides in 4.1^{68/65} presumably represent fusion peptides of the 16- and 24-kD domains, the result of deletion of part or all of the intervening 10 kD spectrin-actin binding domain. These studies did not identify the ~ 3-kD difference between the two LMW mutants.

Mechanical stability of erythrocyte membranes. Ektacytometric measurement of mechanical stability of membranes derived from erythrocytes with the high and low molecular variants of 4.1, as well as membranes with reduced content of normal 4.1 is shown in Fig. 7a. The rate of decline of deformability index (a measure of mechanical stability) of membranes with high molecular weight 4.1 was identical to that of normal membranes, implying that these membranes exhibit normal mechanical stability. In contrast, the deformability index of membranes with low molecular weight 4.1 declined at a much faster rate, implying decreased mechanical stability. The observed decrease in mechanical stability of these membranes was comparable to that of membranes with a 50% re-

duction in membrane content of normal 4.1. These data imply that, while the high molecular variant (4.1⁹⁵) functions normally in bestowing mechanical stability to the membrane, the low molecular weight variant (4.1^{68/65}) is dysfunctional.

To assess if normal mechanical stability could be restored to unstable membranes with a low molecular weight variant of 4.1, we reconstituted these membranes with purified normal 4.1. As shown in Fig. 7 b, as increasing concentrations of purified normal 4.1 were used for reconstitution, the mechanical stability progressively improved. Maximal increase in mechanical stability was seen at a protein concentration of 220 µg/ml, when the mechanical stability of the reconstituted membranes increased twofold. Further increases in concentration of normal 4.1 used during reconstitution did not result in further improvement in mechanical stability. This is in contrast to membranes with quantitative deficiency of protein 4.1, in which complete normalization of mechanical stability could be achieved following reconstitution with normal 4.1 (3).

Discussion

Homozygous deficiency of 4.1 in the erythrocytes of three offspring of a consanguineous marriage resulted in severe hemolytic elliptocytosis demonstrating an important role for 4.1 in maintaining normal shape, membrane mechanical stability and lifespan (1-3). Since this original description of an association between 4.1 and hereditary elliptocytosis, a number of additional families have been identified in which elliptocytic phenotype was linked to either a qualitative defect or a quantitative deficiency of protein 4.1 (4, 7, 20-22). However, no information is currently available regarding the biochemical defect or structural sequelae of these mutant 4.1 proteins.

Our strategy for the characterization of the mutant 4.1 molecules was based on the approach used by Leto et al. for structural characterization of normal 4.1⁸⁰ (8, 14, 15). To locate sites of insertion or deletion, peptides of normal 4.1⁸⁰ and the mutant 4.1⁹⁵ and 4.1^{68/65} produced by enzymatic and chemical cleavages were mapped in one or two dimensions and analyzed by immunochemical analysis using antibodies to five synthetic peptides that span the entire normal 4.1 molecule. These data and NH₂-terminal sequencing of a small chymotryptic peptide (~ 12 kD) unique to 4.1⁹⁵ suggested that the insertion involved repeated sequence of the 24-kD domain, located anomalously NH₂-terminal to the 10-kD spectrin-actin binding domain. However, the size of the 4.1⁹⁵ indicated that the insertion involved more than repeat of ~ 6-kD of the 24-kD domain, and the preservation of its chymotryptic domain structure suggested that the entire insertion consisted

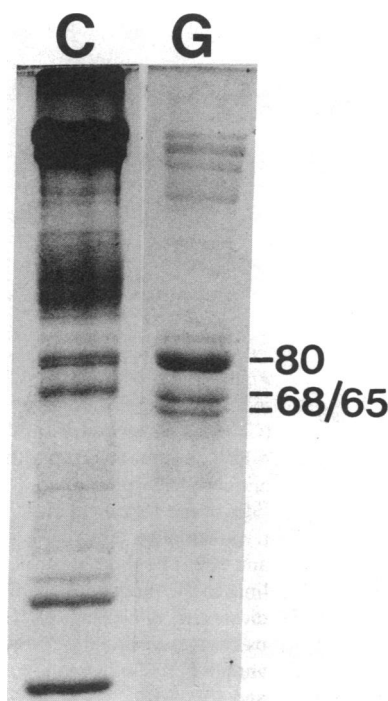


Figure 5. Protein 4.1 purified from red cells of patient G (heterozygous for low molecular weight form of protein 4.1) and normal red cells (C). Numbers indicate apparent kilodaltons of 4.1 proteins.

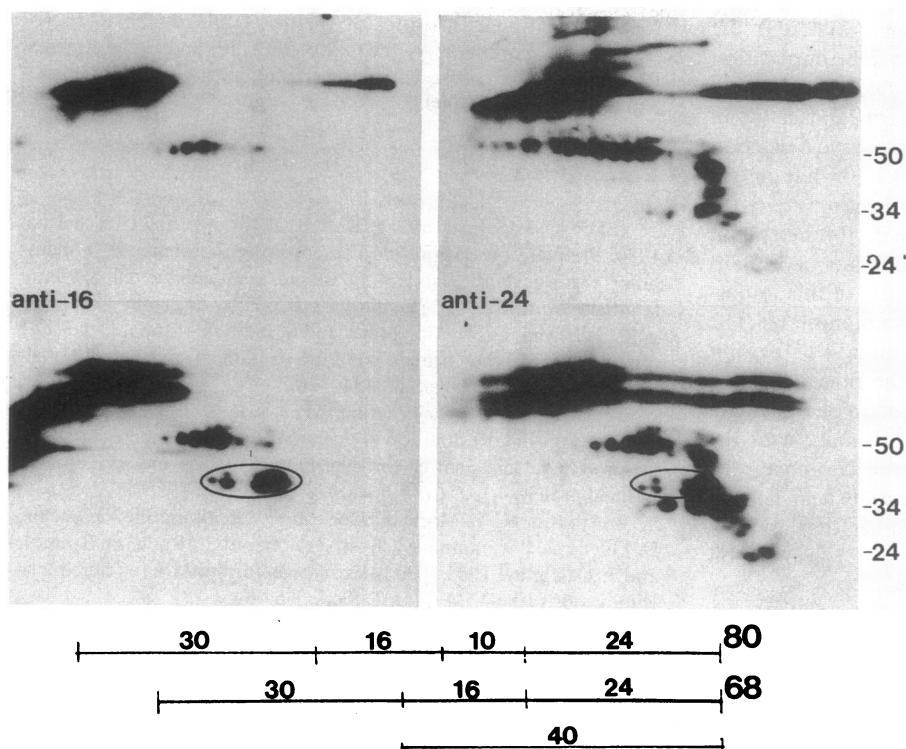


Figure 6. Chymotryptic maps of 4.1^{80} and $4.1^{80+68/65}$ immunoblotted with antibodies to synthetic peptides of the 16- and 24-kD chymotryptic domains of 4.1. Numbers at the right indicate apparent kilodaltons of peptides. Circled peptides are unique to $4.1^{80+68/65}$. The drawing below suggests the boundaries of the new 40-kD peptides.

of repeated sequence. The small size (12–17 kD) of the 10a reactive CNBr fragments unique to 4.1^{95} and the position of available methionines for cleavage, further suggested that the repeat included all or part of the 10-kD domain. The LMW variant, $4.1^{68/65}$, was found to lack most or all of the spectrin-

actin binding domain. The precise boundaries of the insertion in 4.1^{95} and deletion in $4.1^{68/65}$ have been defined by molecular cloning of mutant mRNA, sequencing using PCR techniques, and these data are described in a companion paper.

It is noteworthy that both the mutations we have character-

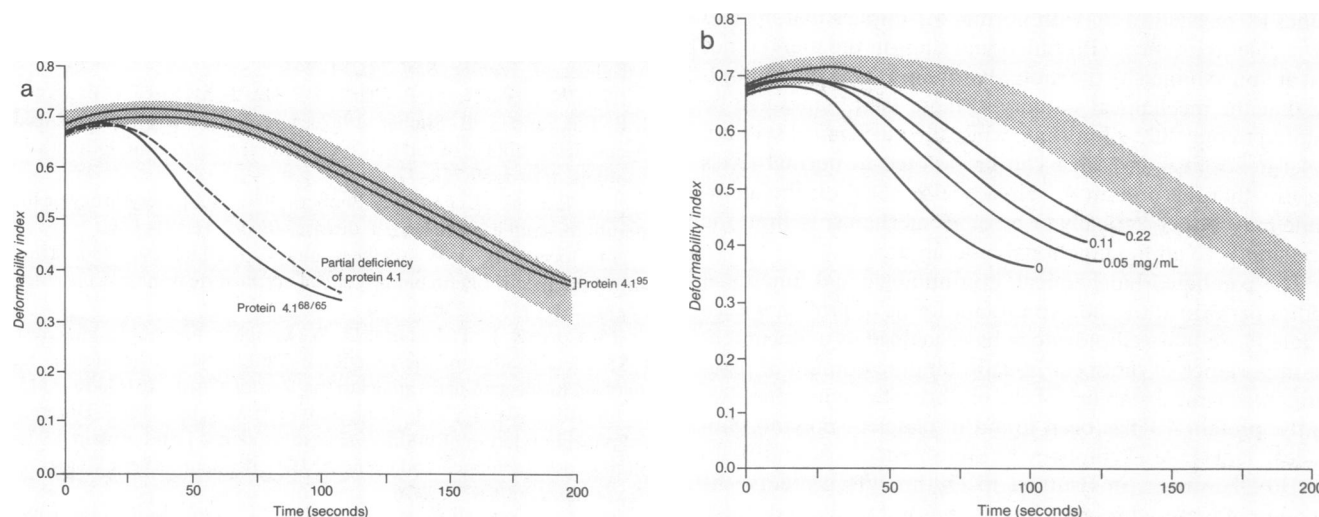


Figure 7. (a) Mechanical stability of erythrocyte membranes. Resealed ghosts prepared from normal and mutant protein 4.1 cells were exposed to 750 dyns/cm² in the ektacytometer and decline of the deformability index (DI) was measured as a function of time. The rate of DI decline is a measure of membrane mechanical stability. Membranes of cells with high molecular weight variant of 4.1 (4.1^{95}) fragmented normally, while membranes of cells with low molecular weight variant ($4.1^{68/65}$) fragmented more rapidly than did normal membranes. Membranes partially deficient in normal 4.1 also fragmented at a rate similar to that of membranes with a low molecular variant. (b) Restoration of mechanical stability to membranes with a low molecular weight 4.1 variant. Ghosts prepared from these cells were incubated with 0, 0.05, 0.11, and 0.22 mg/ml of normal 4.1 before resealing. Increasing the concentration of normal 4.1 resulted in a decrease in the rate of fragmentation, implying improved mechanical stability.

ized involve the spectrin-actin binding domain of 4.1. In the high molecular weight variant, there appears to be duplication of this important functional domain, resulting in 4.1⁹⁵ having two binding sites for interaction with spectrin and actin. In contrast, the low molecular variant appears to have a deletion of this functional domain, resulting in 4.1^{68/65} having no binding site for interaction with spectrin and actin.

The structural and functional changes in erythrocytes with alterations in the spectrin-actin binding domain of mutant 4.1's have enabled us to elucidate the function of this important structural component of the erythrocyte membrane. The finding that erythrocyte membranes containing 4.1⁹⁵ exhibit normal mechanical stability implies that the presence of an additional binding domain for spectrin and actin on this molecule does not alter its ability to interact normally with the other structural components of the membrane. Moreover, the absence of fragmented cells in circulation and the near-normal hematologic status of these individuals implies that erythrocytes with a high molecular weight variant of 4.1 also exhibit normal function in vivo.

These findings are in marked contrast to those seen with erythrocytes with a low molecular weight variant of 4.1. The mechanical stability of membranes of these cells is markedly decreased. In fact, our finding that the mechanical stability of these membranes is very similar to that of membranes with a 50% reduction in 4.1 content implies that 4.1^{68/65} is unable to bestow any mechanical integrity to the membrane. These data thus enable us to identify an important role for spectrin-actin binding domain of protein 4.1 in regulating mechanical stability of the erythrocyte membrane. The presence of fragmented cells in the circulation and the identification of a moderately severe hemolytic anemia in these individuals further implies that the erythrocytes in which 4.1 lacks the spectrin-actin binding domain are also functionally defective in vivo. Our ability to improve the mechanical stability of unstable 4.1^{68/65} membranes by reconstitution with normal 4.1 implies that normal 4.1 is able to interact with functional sites on the membranes that are unoccupied by the mutant protein. However, our finding that the mechanical stability of these membranes can be restored to only 80% of normal, while the mechanical stability of 4.1-deficient membranes can be restored to normal levels suggests that the mutant 4.1 already assembled on the membrane may, either sterically or by other mechanisms, limit the accessibility to all the functional binding sites.

The combined biochemical, immunologic and functional studies performed on the two mutant forms of protein 4.1-associated hereditary elliptocytosis have enabled us to document the importance of the spectrin-actin binding domain in regulating mechanical stability of the erythrocyte membrane. Recently, protein 4.1 has been found in platelets, polymorphonuclear leukocytes, lymphocytes, fibroblasts, lens, and brain (23-30). However, in contrast to erythrocyte proteins, the function of nonerythroid protein 4.1 has not yet been defined. Studies on nonerythroid protein 4.1 isolated from cells of our patients with mutant proteins might shed light on the role of this structural protein in these cells.

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